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UNITED STATES PATENT APPLICATION

OF

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FOR

METHOD FOR TREATING FIBROSIS

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Description

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METHOD OF TREATING FIBROSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. 119(e) from U.S. provisional applications Serial Nos. 60/161,653, filed October 21, 1999; 60/165,255,  
10 filed November 12, 1999; and 60/222,223, filed August 1, 2000.

BACKGROUND OF THE INVENTION

Fibrosis is the abnormal accumulation of fibrous tissue that can occur as a part of the wound-healing process in damaged tissue. Such tissue damage may result  
15 from physical injury, inflammation, infection, exposure to toxins, and other causes. Examples of fibrosis include dermal scar formation, keloids, liver fibrosis, lung fibrosis (e.g., silicosis, asbestosis), kidney fibrosis (including diabetic nephropathy), and glomerulosclerosis.

Liver (hepatic) fibrosis, for example, occurs as a part of the wound-  
20 healing response to chronic liver injury. Fibrosis occurs as a complication of haemochromatosis, Wilson's disease, alcoholism, schistosomiasis, viral hepatitis, bile duct obstruction, exposure to toxins, and metabolic disorders. This formation of scar tissue is believed to represent an attempt by the body to encapsulate the injured tissue. Liver fibrosis is characterized by the accumulation of extracellular matrix that can be  
25 distinguished qualitatively from that in normal liver. Left unchecked, hepatic fibrosis progresses to cirrhosis (defined by the presence of encapsulated nodules), liver failure, and death.

In recent years there have been significant advances in the understanding of the cellular and biochemical mechanisms underlying liver fibrosis (reviewed by Li  
30 and Friedman, *J. Gastroenterol. Hepatol.* 14:618-633, 1999). Stellate cells are believed to be a major source of extracellular matrix in the liver. Stellate cells respond to a variety of cytokines present in the liver, some of which they also produce (Friedman, *Seminars in Liver Disease* 19:129-140, 1999).

As summarized by Li and Friedman (*ibid.*), actual and proposed  
35 therapeutic strategies for liver fibrosis include removal of the underlying cause (e.g., toxin or infectious agent), suppression of inflammation (using, e.g., corticosteroids, IL-1 receptor antagonists, or other agents), down-regulation of stellate cell activation

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(using, e.g., gamma interferon or antioxidants), promotion of matrix degradation, or promotion of stellate cell apoptosis. Despite recent progress, many of these strategies are still in the experimental stage, and existing therapies are aimed at suppressing inflammation rather than addressing the underlying biochemical processes. Thus, there remains a need in the art for materials and methods for treating fibrosis, including liver fibrosis.

## DESCRIPTION OF THE INVENTION

The present invention provides materials and methods for reducing cell proliferation or extracellular matrix production, treating fibrosis, and reducing stellate cell activation in a mammal.

Within one aspect of the invention there is provided a method of reducing cell proliferation or extracellular matrix production in a mammal comprising administering to the mammal a composition comprising a zvegf3 antagonist in combination with a pharmaceutically acceptable delivery vehicle, wherein the zvegf3 antagonist is selected from the group consisting of anti-zvegf3 antibodies, mitogenically inactive receptor-binding zvegf3 variant polypeptides, and inhibitory polynucleotides, in an amount sufficient to reduce cell proliferation or extracellular matrix production. Within certain embodiments of the invention, proliferation of mesangial, endothelial, smooth muscle, fibroblast, osteoblast, osteoclast, stellate, or interstitial cells is reduced. Within other embodiments, the mammal is suffering from a fibroproliferative disorder of the liver, kidney, or bone.

Within another aspect of the present invention there is provided a method of treating fibrosis in a mammal comprising administering to the mammal a composition comprising a therapeutically effective amount of a zvegf3 antagonist in combination with a pharmaceutically acceptable delivery vehicle, wherein the zvegf3 antagonist is selected from the group consisting of anti-zvegf3 antibodies, mitogenically inactive receptor-binding zvegf3 variant polypeptides, and inhibitory polynucleotides. Within certain embodiment of the invention the fibrosis is liver fibrosis or kidney fibrosis.

Within a third aspect of the invention there is provided a method of reducing stellate cell activation in a mammal comprising administering to the mammal a composition comprising a zvegf3 antagonist in combination with a pharmaceutically acceptable delivery vehicle, wherein the zvegf3 antagonist is selected from the group consisting of anti-zvegf3 antibodies, mitogenically inactive receptor-binding zvegf3 variant polypeptides, and inhibitory polynucleotides, in an amount sufficient to reduce stellate cell activation. Within one embodiment, the stellate cells are liver stellate cells.

Within a fourth aspect of the invention there are provided pharmaceutical compositions for use within the above methods. In general, the compositions comprise a zveg3 antagonist in combination with a pharmaceutically acceptable delivery vehicle, wherein the zveg3 antagonist is selected from the group consisting of anti-zveg3 antibodies, mitogenically inactive receptor-binding zveg3 variant polypeptides, and inhibitory polynucleotides.

These and other aspects of the invention will become evident upon reference to the following detailed description of the invention and the attached drawings. In the drawings:

Fig. 1 is a Hopp/Woods hydrophilicity profile of the amino acid sequence shown in SEQ ID NO:2. The profile is based on a sliding six-residue window. Buried G, S, and T residues and exposed H, Y, and W residues were ignored. These residues are indicated in the figure by lower case letters.

Fig. 2 is an alignment of human (SEQ ID NO:2) and mouse (SEQ ID NO:4) amino acid sequences.

The term "antagonist" is used herein to denote a compound that reduces a biological activity of another compound. Within the present invention, a "zveg3 antagonist" is a compound that reduces the receptor-mediated biological activity (e.g., mitogenic activity) of zveg3 on a target cell. Antagonists may exert their action by competing with zveg3 for binding sites on a cell-surface receptor, by binding to zveg3 and preventing it from binding to a cell-surface receptor, by otherwise interfering with receptor function, by reducing production of zveg3, or by other means.

"Extracellular matrix" (ECM) is a complex mixture of macromolecules that accumulates within tissues in close apposition to cell surfaces. ECM contains secreted macromolecules such as collagens I, III and IV, fibronectin, laminins, and various proteoglycans. These macromolecules can be organized to provide cohesion to the tissue and can contribute to its structural and mechanical properties. ECM can act as a depository for, and release site of, potent secreted growth factors, and is known to influence growth, survival and differentiation of the cells it surrounds. Pathologic ECM accumulation, if unchecked, can restrict access of nutrients, growth factors and other physiologically important molecules to cells and can lead to the creation of areas of low live cell density. Over time, this accumulation can result in the inability of a tissue to perform its specific metabolic and structural roles, and may ultimately lead to overt cell and tissue death.

An "inhibitory polynucleotide" is a DNA or RNA molecule that reduces or prevents expression (transcription or translation) of a second (target) polynucleotide. Inhibitory polynucleotides include antisense polynucleotides, ribozymes, and external

guide sequences. The term "inhibitory polynucleotide" further includes DNA and RNA molecules that encode the actual inhibitory species, such as DNA molecules that encode ribozymes.

The terms "treat" and "treatment" are used broadly to denote therapeutic and prophylactic interventions that favorably alter a pathological state. Treatments include procedures that moderate or reverse the progression of, reduce the severity of, prevent, or cure a disease.

All references cited herein are incorporated by reference in their entirety.

The present invention provides methods for treating fibrosis in a patient using zveg3 antagonists. Zveg3 is a protein that is structurally related to platelet-derived growth factor (PDGF) and the vascular endothelial growth factors (VEGF). This protein has also been designated "VEGF-R" (WIPO Publication WO 99/37671) and, more recently, "PDGF-C" (WO 00/18212). Zveg3/PDGF-C is a multi-domain protein with significant homology to the PDGF/VEGF family of growth factors. Representative amino acid sequences of human and mouse zveg3 are shown in SEQ ID NO:2 and SEQ ID NO:4, respectively. DNAs encoding these polypeptides are shown in SEQ ID NOS:1 and 3, respectively.

The term "zveg3 protein" is used herein to denote proteins comprising the growth factor domain of a zveg3 polypeptide (e.g., residues 235-345 of human zveg3 (SEQ ID NO:2) or mouse zveg3 (SEQ ID NO:4)), wherein said protein is mitogenic for cells expressing cell-surface PDGF  $\alpha$ -receptor subunit. Zveg3 has been found to bind to the  $\alpha\alpha$  and  $\alpha\beta$  isoforms of PDGF receptor. Zveg3 proteins include homodimers and heterodimers as disclosed below. Using methods known in the art, zveg3 proteins can be prepared in a variety of forms, including glycosylated or non-glycosylated, pegylated or non-pegylated, with or without an initial methionine residues, and as fusion proteins as disclosed in more detail below.

Structural predictions based on the zveg3 sequence and its homology to other growth factors suggests that the polypeptide can form homomultimers or heteromultimers having growth factor activity, i.e., modulating one or more of cell proliferation, migration, differentiation, and metabolism. Experimental evidence confirms that biologically active zveg3 is a dimeric protein. While not wishing to be bound by theory, the similarity of zveg3 to other members of the PDGF/VEGF family suggests that zveg3 may also form heteromultimers with other members of the family, including VEGF, VEGF-B, VEGF-C, VEGF-D, zveg4 (SEQ ID NO:5), PIGF (Maglione et al., *Proc. Natl. Acad. Sci. USA* 88:9267-9271, 1991), PDGF-A (Murray et

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al., U.S. Patent No. 4,899,919; Heldin et al., U.S. Patent No. 5,219,759), or PDGF-B (Chiu et al., *Cell* 37:123-129, 1984; Johnsson et al., *EMBO J.* 3:921-928, 1984).

The zveg3 polypeptide chain comprises a growth factor domain and a CUB domain. The growth factor domain is characterized by an arrangement of cysteine residues and beta strands that is characteristic of the "cystine knot" structure of the PDGF family. The CUB domain shows sequence homology to CUB domains in the neuropilins (Takagi et al., *Neuron* 7:295-307, 1991; Soker et al., *ibid.*), human bone morphogenetic protein-1 (Wozney et al., *Science* 242:1528-1534, 1988), porcine seminal plasma protein and bovine acidic seminal fluid protein (Romero et al., *Nat. Struct. Biol.* 4:783-788, 1997), and *X. laevis* tolloid-like protein (Lin et al., *Dev. Growth Differ.* 39:43-51, 1997).

An alignment of mouse and human zveg3 polypeptide sequences is shown in Fig. 2. Analysis of the amino acid sequence shown in SEQ ID NO:2 indicates that residues 1 to 14 form a secretory peptide. The CUB domain extends from residue 46 to residue 163. A propeptide-like sequence extends from residue 164 to residue 234, and includes two potential cleavage sites at its carboxyl terminus, a dibasic site at residues 231-232 and a target site for furin or a furin-like protease at residues 231-234. The growth factor domain extends from residue 235 to residue 345. Those skilled in the art will recognize that domain boundaries are somewhat imprecise and can be expected to vary by up to  $\pm 5$  residues from the specified positions. Potential proteolytic cleavage sites occur at residues 232 and 234. Processing of recombinant zveg3 produced in BHK cells has been found to occur between residues 225 and 226. Signal peptide cleavage is predicted to occur after residue 14 ( $\pm 3$  residues). This analysis suggests that the zveg3 polypeptide chain may be cleaved to produce a plurality of monomeric species as shown in Table 1. Cleavage after Arg-234 is expected to result in subsequent removal of residues 231-234, with possible conversion of Gly-230 to an amide. Cleavage after Lys-232 is expected to result in subsequent removal of residue 231, again with possible conversion of Gly-230 to an amide. In addition, it may be advantageous to include up to seven residues of the interdomain region at the carboxyl terminus of the CUB domain. The interdomain region can be truncated at its amino terminus by a like amount. See Table 1. Corresponding domains in mouse and other non-human zveg3s can be determined by those of ordinary skill in the art from sequence alignments.

Table 1

<u>Monomer</u>	<u>Residues (SEQ ID NO:2)</u>
Cub domain	15 - 163 46 - 163 15 - 170 46 - 170
CUB domain + interdomain region	15 - 234 46 - 234 15 - 229 amide 15 - 230
Cub domain + interdomain region + growth factor domain	15 - 345 46 - 345
Growth factor domain	235 - 345 226 - 345
Growth factor domain + interdomain region	164 - 345 171 - 345

Zveg3 can thus be prepared in a variety of multimeric forms comprising a zveg3 polypeptide as disclosed above. These zveg3 polypeptides include zveg3<sub>15-345</sub>, zveg3<sub>46-345</sub>, zveg3<sub>226-345</sub>, and zveg3<sub>235-345</sub>. Variants and derivatives of these polypeptides can also be prepared as disclosed herein.

Zveg3 proteins can be prepared as fusion proteins comprising amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, an affinity tag, or a targetting polypeptide. For example, a zveg3 protein can be prepared as a fusion with an affinity tag to facilitate purification. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include, for example, a poly-histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-4, 1985), substance P, Flag<sup>TM</sup> peptide (Hopp et al., *Biotechnology* 6:1204-1210, 1988), streptavidin binding peptide,

maltose binding protein (Guan et al., *Gene* 67:21-30, 1987), cellulose binding protein, thioredoxin, ubiquitin, T7 polymerase, or other antigenic epitope or binding domain. Fusion of zveg3 to, for example, maltose binding protein or glutathione S transferase can be used to improve yield in bacterial expression systems. In these instances the

5 non-zveg3 portion of the fusion protein ordinarily will be removed prior to use. Separation of the zveg3 and non-zveg3 portions of the fusion protein is facilitated by providing a specific cleavage site between the two portions. Such methods are well known in the art. Zveg3 can also be fused to a targeting peptide, such as an antibody (including polyclonal antibodies, monoclonal antibodies, antigen-binding fragments thereof such as F(ab')<sub>2</sub> and Fab fragments, single chain antibodies, and the like) or

10 other peptidic moiety that binds to a target tissue.

Variations can be made in the zveg3 amino acid sequences shown in SEQ ID NO:2 and SEQ ID NO:4 to provide mitogenically inactive, receptor-binding polypeptides that act as zveg3 antagonists. As used herein, the term “mitogenically

15 inactive” means that the protein does not show statistically significant activity in a standard mitogenesis assay as compared to a wild-type zveg3 control. Such variations include amino acid substitutions, deletions, and insertions. While not wishing to be bound by theory, it is believed that residues Arg260-Trp271 of human zveg3 (SEQ ID NO:2) form a loop that define the ability of the protein to bind to PDGF- $\beta$  receptors, although binding is also permitted to alpha receptors. It is thus predicted that binding

20 to either receptor subunit can be blocked or enhanced by mutations in this region. In addition, residues Leu311-His321 of SEQ ID NO:2 are predicted to form a loop (loop3) that may be mutated to block receptor binding. Peptides that mimic this region of the molecule may act as antagonists.

The effects of amino acid sequence changes can be predicted by

25 computer modeling (using, e.g., the Insight II® viewer and homology modeling tools; MSI, San Diego, CA) or determined by analysis of crystal structure (see, e.g., Laphorn et al., *Nature* 369:455, 1994), and can be assessed using art-recognized mutagenesis procedures in combination with activity assays. Representative mutagenesis

30 procedures include, for example, site-directed mutagenesis and alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244, 1081-1085, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-4502, 1991). Multiple amino acid substitutions can be made and tested using known methods, such as those disclosed by Reidhaar-Olson and Sauer (*Science* 241:53-57, 1988) or Bowie and Sauer (*Proc. Natl. Acad. Sci. USA*

35 86:2152-2156, 1989). Other methods that can be used include phage display (e.g., Lowman et al., *Biochem.* 30:10832-10837, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204), region-directed mutagenesis

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(Derbyshire et al., *Gene* 46:145, 1986; Ner et al., *DNA* 7:127, 1988), and DNA shuffling as disclosed by Stemmer (*Nature* 370:389-391, 1994) and Stemmer (*Proc. Natl. Acad. Sci. USA* 91:10747-10751, 1994). The resultant mutant molecules are tested for mitogenic activity or other properties (e.g., receptor binding) to identify amino acid residues that are critical to these functions. Mutagenesis can be combined with high volume or high-throughput screening methods to detect biological activity of zveg3 variant polypeptides, in particular biological activity in modulating cell proliferation. For example, mitogenesis assays that measure dye incorporation or <sup>3</sup>H-thymidine incorporation can be carried out on large numbers of samples. Competition assays can be employed to confirm antagonist activity.

Zveg3 proteins, including full-length polypeptides, fragments, and fusion proteins, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells (including cultured cells of multicellular organisms). Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and Ausubel et al., eds., *Current Protocols in Molecular Biology*, Green and Wiley and Sons, NY, 1993. See, WO 00/34474. In general, a DNA sequence encoding a zveg3 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers. See, WO 00/34474.

Zveg3 proteins can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipercolic acid, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-

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fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., *J. Am. Chem. Soc.* 113:2722, 1991; Ellman et al., *Methods Enzymol.* 202:301, 1991; Chung et al., *Science* 259:806-809, 1993; and Chung et al., *Proc. Natl. Acad. Sci. USA* 90:10145-10149, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., *J. Biol. Chem.* 271:19991-19998, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., *Biochem.* 33:7470-7476, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403, 1993).

Zveg3 polypeptides or fragments thereof can also be prepared through chemical synthesis according to methods known in the art, including exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. See, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149, 1963; Stewart et al., Solid Phase Peptide Synthesis (2nd edition), Pierce Chemical Co., Rockford, IL, 1984; Bayer and Rapp, *Chem. Pept. Prot.* 3:3, 1986; and Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford, 1989.

Zveg3 proteins are purified by conventional protein purification methods, typically by a combination of chromatographic techniques. See, in general, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988; and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York, 1994. Proteins comprising a polyhistidine affinity tag (typically about 6 histidine residues) are purified by affinity chromatography on a nickel chelate resin. See, for example, Houchuli et al., *Bio/Technol.* 6: 1321-1325, 1988. Furthermore, the growth factor domain itself binds to nickel resin at pH 7.0-8.0

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and 25 mM Na phosphate, 0.25 M NaCl. Bound protein can be eluted with a descending pH gradient down to pH 5.0 or an imidazole gradient. Recombinant zveg3 growth factor domain protein can be purified using a combination of chromatography on a strong cation exchanger followed by a tandem column array comprising a strong anion exchanger followed by an immobilized metal affinity column in series. It has also been found that zveg3 binds to various dye matrices (e.g., BLUE1, BLUE 2, ORANGE 1, ORANGE 3, and RED3 from Lexton Scientific, Signal Hill, CA) in PBS at pH 6-8, from which the bound protein can be eluted in 1-2 M NaCl in 20 mM boric acid buffer at pH 8.8. Protein eluted from RED3 may be passed over RED2 (Lexton Scientific) to remove remaining contaminants. Proteins comprising a glu-glu tag can be purified by immunoaffinity chromatography according to conventional procedures. See, for example, Grussenmeyer et al., *ibid.* Maltose binding protein fusions are purified on an amylose column according to methods known in the art.

As disclosed in more detail below, overexpression of zveg3 in the livers of transgenic mice led to marked stellate cell activation and proliferation. At 8 weeks of age there was an accumulation of perisinusoidal extracellular matrix (ECM) that progressed to a perivenular ECM deposition at 22 weeks and possible early stages of cirrhosis characterized by fibrotic banding and hepatic nodule formation at 33 weeks of age. Thus, zveg3 dimers appear to resemble the previously described PDGF isoforms in being potent mitogens of hepatic stellate cells and appearing to play a role in liver fibrosis. These transgenic mice thus provide a model for testing zveg3 antagonists as well as other antifibrotic agents. In view of these and other experiments disclosed herein, it is expected that altered zveg3 expression may initiate or exacerbate a variety of fibrotic conditions. In this context, inhibiting the action of zveg3 using a zveg3 antagonist will limit the progress of such conditions. While not wishing to be bound by theory, it is believed that the pro-fibrotic effects of zveg3 are due at least in part to the induction of TGF- $\beta$  production.

Zveg3 antagonists include, without limitation, anti-zveg3 antibodies (including neutralizing antibodies), inhibitory polynucleotides (including antisense polynucleotides, ribozymes, and external guide sequences), and other peptidic and non-peptidic agents, including small molecule inhibitors and mitogenically inactive receptor-binding zveg3 polypeptides. Such antagonists can be used to block the mitogenic effects of zveg3 and thereby reduce, inhibit, prevent, or otherwise treat fibrosis, including, without limitation, scar formation, keloids, scleroderma, liver fibrosis, lung fibrosis, kidney fibrosis, pancreatic fibrosis, myelofibrosis, post-surgical fibrotic adhesions, fibroproliferative disorders of the vasculature, fibroproliferative



and Ooi, *J. Immunol.* 151:2247-2251, 1993). Infection by hepatitis-C virus can also result in idiopathic membranoproliferative glomerulonephritis (Johnson et al., *N. Engl. J. Med.* 328:465-470, 1993). While not wishing to be bound by theory, experiments have shown that the activity of zveg3 is mediated by the  $\alpha\alpha$  and  $\alpha\beta$  PDGF receptor isoforms. PDGF receptors are widely expressed in most renal cell types, and their expression is upregulated in a number of kidney pathologies (e.g., Iida et al., *Proc. Natl. Acad. Sci. USA* 88:6560-6564, 1991). Stimulation of PDGF receptors has been implicated in fibroproliferative diseases of the kidney in a variety of animal models (e.g., Ooi et al., *P.S.E.B.M.* 213:230-237, 1996; Lindahl et al., *Development* 125:3313-3322, 1998; Lindahl and Betsholtz, *Curr. Op. Nephrol. Hypert.* 7:21-26, 1998; and Betsholtz and Raines, *Kidney Int.* 51:1361-1369, 1997).

Fibrotic disorders of the lung include, for example, silicosis, asbestosis, idiopathic pulmonary fibrosis, bronchiolitis obliterans-organizing pneumonia, pulmonary fibrosis associated with high-dose chemotherapy, idiopathic pulmonary fibrosis, and pulmonary hypertension. These diseases are characterized by cell proliferation and increased production of extracellular matrix components, such as collagens, elastin, fibronectin, and tenascin-C.

Pancreatic fibrosis occurs in chronic pancreatitis. This condition is characterized by duct calcification and fibrosis of the pancreatic parenchyma. Like liver cirrhosis, chronic pancreatitis is associated with alcohol abuse. See, Fogar et al., *J. Medicine* 29:277-287, 1998.

Diseases of the skeleton that are due to modified growth and matrix production in the bone include, but are not limited to, osteopetrosis, hyperostosis, osteosclerosis, osteoarthritis, and endosteal bone formation in metastatic prostate cancer. Fibroproliferative disorders of bone are characterized by aberrant and ectopic bone formation, commonly seen as active proliferation of the major cell types participating in bone formation as well as elaboration by those cells of a complex bone matrix. Exemplary of such bone disorders is the fibrosis that occurs with prostate tumor metastases to the axial skeleton. In prostate tumor-related cancellous bone growth, prostate carcinoma cells can interact reciprocally with osteoblasts to produce enhanced tumor growth and osteoblastic action when they are deposited in bone (Zhau et al., *Cancer* 88:2995-3001, 2000; Ritchie et al., *Endocrinology* 138:1145-1150, 1997). Fibroproliferative responses of the bone originating in the skeleton *per se* include osteopetrosis and hyperostosis. A defect in osteoblast differentiation and function is thought to be a major cause in osteopetrosis, an inherited disorder characterized by bone sclerosis due to reduced bone resorption, marrow cavities fail to develop, resulting in extramedullary hematopoiesis and severe hematologic abnormalities associated with

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optic atrophy, deafness, and mental retardation (Lajeunesse et al., *J. Clin. Invest.* 98:1835-1842, 1996). In osteoarthritis, bone changes are known to occur, and bone collagen metabolism is increased within osteoarthritic femoral heads. The greatest changes occur within the subchondral zone, supporting a greater proportion of osteoid in the diseased tissue (Mansell and Bailey, *J. Clin. Invest.* 101:1596-1603, 1998). As shown in more detail below, zveg3 has been found to be produced by prostate cells and to stimulate an osteoblast cell line.

Fibroproliferative disorders of the vasculature include, for example, transplant vasculopathy, which is a major cause of chronic rejection of heart transplantation. Transplant vasculopathy is characterized by accelerated atherosclerotic plaque formation with diffuse occlusion of the coronary arteries, which is a "classic" fibroproliferative disease. See, Miller et al., *Circulation* 101:1598-1605, 2000).

Antibodies used as zveg3 antagonists include antibodies that specifically bind to a zveg3 protein and, by so binding, reduce or prevent the binding of zveg3 protein to the receptor and, consequently, reduce or block the receptor-mediated activity of zveg3. As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')<sub>2</sub> and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Monoclonal antibodies can also be produced in mice that have been genetically altered to produce antibodies that have a human structure.

Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, Cooligan et al. (eds.), Current Protocols in Immunology, National Institutes of Health, John Wiley and Sons, Inc., 1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor, NY, 1989; and Hurrell (ed.), Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982. As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated by

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inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a zveg3 polypeptide or a fragment thereof.

Immunogenic polypeptides will comprise an epitope-bearing portion of a zveg3 polypeptide (e.g., as shown in SEQ ID NO:2) or receptor. An "epitope" is a region of a protein to which an antibody can bind. See, for example, Geysen et al., *Proc. Natl. Acad. Sci. USA* 81:3998-4002, 1984. Epitopes can be linear or conformational, the latter being composed of discontinuous regions of the protein that form an epitope upon folding of the protein. Linear epitopes are generally at least 6 amino acid residues in length. Relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, Sutcliffe et al., *Science* 219:660-666, 1983. Immunogenic, epitope-bearing polypeptides contain a sequence of at least six, often at least nine, more often from 15 to about 30 contiguous amino acid residues of a zveg3 protein. Polypeptides comprising a larger portion of a zveg3 protein, i.e. from 30 to 50 residues up to the entire sequence are included. It is preferred that the amino acid sequence of the epitope-bearing polypeptide is selected to provide substantial solubility in aqueous solvents, that is the sequence includes relatively hydrophilic residues, and hydrophobic residues are substantially avoided. Such regions include residues 43-48, 96-101, 97-102, 260-265, and 330-335 of SEQ ID NO:2. As noted above, it is generally preferred to use somewhat longer peptides as immunogens, such as a peptide comprising residues 80-104, 299-314, and 299-326 of SEQ ID NO:2. The latter peptide can be prepared with an additional N-terminal Cys residue to facilitate coupling.

The immunogenicity of a polypeptide immunogen may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of a zveg3 polypeptide or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), or tetanus toxoid) for immunization.

Alternative techniques for generating or selecting antibodies include *in vitro* exposure of lymphocytes to a polypeptide immunogen, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled polypeptide). Techniques for creating and screening such random peptide display libraries are known in the art (e.g., Ladner et al., US Patent No. 5,223,409; Ladner et al., US Patent No. 4,946,778; Ladner et al., US Patent No. 5,403,484 and

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Ladner et al., US Patent No. 5,571,698), and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech Laboratories (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ).

- 5 Random peptide display libraries can be screened using the zveg3 sequences disclosed herein to identify proteins which bind to zveg3.

Antibodies are determined to be specifically binding if they bind to their intended target (e.g., zveg3 protein or receptor) with an affinity at least 10-fold greater than the binding affinity to control (e.g., non-zveg3 or non-receptor) polypeptide or  
 10 protein. In this regard, a "non-zveg3 polypeptide" includes the related molecules VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF-A, and PDGF-B, but excludes zveg3 polypeptides from non-human species. Due to the high level of amino acid sequence identity expected between zveg3 orthologs, antibodies specific for human zveg3 may also bind to zveg3 from other species. The binding affinity of an antibody  
 15 can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, G., *Ann. NY Acad. Sci.* 51: 660-672, 1949). Methods for screening and isolating specific antibodies are well known in the art. See, for example, Paul (ed.), Fundamental Immunology, Raven Press, 1993; Getzoff et al., *Adv. in Immunol.* 43:1-98, 1988; Goding (ed.), Monoclonal Antibodies: Principles and Practice, Academic  
 20 Press Ltd., 1996; Benjamin et al., *Ann. Rev. Immunol.* 2:67-101, 1984.

Binding affinity can also be determined using a commercially available biosensor instrument (BIAcore™, Pharmacia Biosensor, Piscataway, NJ), wherein protein is immobilized onto the surface of a receptor chip. See, Karlsson, *J. Immunol. Methods* 145:229-240, 1991 and Cunningham and Wells, *J. Mol. Biol.* 234:554-563,  
 25 1993. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

A variety of assays known to those skilled in the art can be utilized to detect antibodies that specifically bind to zveg3 proteins or receptors. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane  
 30 (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assays, inhibition or competition assays, and sandwich assays.

For therapeutic applications it is generally preferred to use neutralizing  
 35 antibodies. As used herein, the term "neutralizing antibody" denotes an antibody that inhibits at least 50% of the biological activity of the cognate antigen when the antibody is added at a 1000-fold molar excess. Those of skill in the art will recognize that



greater neutralizing activity is sometimes desirable, and antibodies that provide 50% inhibition at a 100-fold or 10-fold molar excess may be advantageously employed.

5 Zveg3 antagonists further include antisense polynucleotides, which can be used to inhibit zveg3 gene transcription and thereby inhibit cell activation and/or proliferation *in vivo*. Polynucleotides that are complementary to a segment of a zveg3-encoding polynucleotide (e.g., a polynucleotide as set forth in SEQ ID NO:1) are designed to bind to zveg3-encoding mRNA and to inhibit translation of such mRNA. Antisense polynucleotides can be targetted to specific tissues using a gene therapy approach with specific vectors and/or promoters, such as viral delivery systems as disclosed in more detail below.

15 Ribozymes can also be used as zveg3 antagonists within the present invention. Ribozymes are RNA molecules that contain a catalytic center and a target RNA binding portion. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A ribozyme selectively binds to a target RNA molecule through complementary base pairing, bringing the catalytic center into close proximity with the target sequence. The ribozyme then cleaves the target RNA and is released, after which it is able to bind and cleave additional molecules. A nucleic acid molecule that encodes a ribozyme is termed a "ribozyme gene." Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). An expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme.

25 In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode a zveg3 polypeptide. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently cleaved by the cellular ribozyme (see, for example, Altman et al., U.S. Patent No. 5,168,053; Yuan et al., *Science* 263:1269, 1994; Pace et al., WIPO Publication No. WO 96/18733; George et al., WIPO Publication No. WO 96/21731; and Werner et al., WIPO Publication No. WO 97/33991). An external guide sequence generally comprises a ten- to fifteen-nucleotide sequence complementary to zveg3 mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs

between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the base-paired region.

5 The growth factor domain of zveg3 has been found to be the active (PDGF receptor-binding) species of the molecule. Proteolytic processing to remove the N-terminal portion of the molecule is required for activation. Thus, inhibitors of this proteolytic activation can also be used as zveg3 antagonists within the present invention.

10 For pharmaceutical use, zveg3 antagonists are formulated for topical or parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. In general, pharmaceutical formulations will include a zveg3 antagonist in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water, or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss  
15 on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in *Remington: The Science and Practice of Pharmacy*, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995. A "therapeutically effective amount" of a composition is that amount that produces a statistically significant effect, such as a statistically significant reduction in disease progression or a  
20 statistically significant improvement in organ function. The exact dose will be determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The therapeutic formulations will generally be administered over the period required to achieve a beneficial effect,  
25 commonly up to several months and, in treatment of chronic conditions, for a year or more. Dosing is daily or intermittently over the period of treatment. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. Sustained release formulations can also be employed. For treatment of pulmonary fibrosis, a zveg3 antagonist can be delivered by aerosolization according to  
30 methods known in the art. See, for example, Wang et al., US Patent No. 5,011,678; Gonda et al., US Patent No. 5,743,250; and Lloyd et al., US Patent No. 5,960,792.

Other mitogenic factors, including EGF, TGF $\beta$ , and FGF, have been implicated in the initiation or perpetuation of fibrosis. It may therefore be advantageous to combine a zveg3 inhibitor with one or more inhibitors of these other  
35 factors.

Antibodies are preferably administered parenterally, such as by bolus injection or infusion (intravenous, intramuscular, intraperitoneal or subcutaneous) over

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the course of treatment. Antibodies are generally administered in an amount sufficient to provide a minimum circulating level of antibody throughout the treatment period of between approximately 20 µg and 1 mg/kg body weight. In this regard, it is preferred to use antibodies having a circulating half-life of at least 12 hours, preferably at least 4  
5 days, more preferably up to 14-21 days. Chimeric and humanized antibodies are expected to have circulatory half-lives of up to four and up to 14-21 days, respectively. In many cases it will be preferable to administer daily doses during a hospital stay, followed by less frequent bolus injections during a period of outpatient treatment. Antibodies can also be delivered by slow-release delivery systems, pumps, and other  
10 known delivery systems for continuous infusion. Dosing regimens may be varied to provide the desired circulating levels of a particular antibody based on its pharmacokinetics. Thus, doses will be calculated so that the desired circulating level of therapeutic agent is maintained. Daily doses referred to above may be administered as larger, less frequent bolus administrations to provide the recited dose averaged over the  
15 term of administration.

Those skilled in the art will recognize that the same principles will guide the use of other zveg3 antagonists. The dosing regimen for a given antagonist will be determined by a number of factors including potency, pharmacokinetics, and the physicochemical nature of the antagonist. For example, non-peptidic zveg3  
20 antagonists may be administered enterally.

Therapeutic polynucleotides, such as antisense polynucleotides, can be delivered to patients or test animals by way of viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is  
25 currently the best studied gene transfer vector for delivery of heterologous nucleic acids. For review, see Becker et al., *Meth. Cell Biol.* 43:161-89, 1994; and Douglas and Curiel, *Science & Medicine* 4:44-53, 1997. The adenovirus system offers several advantages. Adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used  
30 with many different promoters including ubiquitous, tissue specific, and regulatable promoters. Because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated  
35 into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion,

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the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a signal sequence is present, secrete) the heterologous protein.

An alternative method of gene delivery comprises removing cells from the body and introducing a vector into the cells as a naked DNA plasmid. The transformed cells are then re-implanted in the body. Naked DNA vectors are introduced into host cells by methods known in the art, including transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter. See, Wu et al., *J. Biol. Chem.* 263:14621-14624, 1988; Wu et al., *J. Biol. Chem.* 267:963-967, 1992; and Johnston and Tang, *Meth. Cell Biol.* 43:353-365, 1994.

Activity of zveg3 antagonists can be measured *in vitro* using assays (including cell-based assays) designed to measure zveg3 activity. Antagonists will reduce the effects of zveg3 within the assay. Ligand-receptor binding can be assayed by a variety of methods well known in the art, including receptor competition assays (Bowen-Pope and Ross, *Methods Enzymol.* 109:69-100, 1985) and through the use of soluble receptors, including receptors produced as IgG fusion proteins (U.S. Patent No. 5,750,375). Receptor binding assays can be performed on cell lines that contain known cell-surface receptors for evaluation. The receptors can be naturally present in the cell, or can be recombinant receptors expressed by genetically engineered cells. Mitogenic activity can be measured using known assays, including <sup>3</sup>H-thymidine incorporation assays (as disclosed by, e.g., Raines and Ross, *Methods Enzymol.* 109:749-773, 1985 and Wahl et al., *Mol. Cell Biol.* 8:5016-5025, 1988), dye incorporation assays (as disclosed by, for example, Mosman, *J. Immunol. Meth.* 65:55-63, 1983 and Raz et al., *Acta Trop.* 68:139-147, 1997) or cell counts. Suitable mitogenesis assays measure incorporation of <sup>3</sup>H-thymidine into (1) 20% confluent cultures to look for the ability of zveg3 proteins to further stimulate proliferating cells, and (2) quiescent cells held at confluence for 48 hours to look for the ability of zveg3 proteins to overcome contact-induced growth inhibition. Suitable dye incorporation assays include measurement of the incorporation of the dye Alamar blue (Raz et al., *ibid.*) into target cells. See also, Gospodarowicz et al., *J. Cell. Biol.* 70:395-405, 1976; Ewton and Florini, *Endocrinol.* 106:577-583, 1980; and Gospodarowicz et al., *Proc. Natl. Acad. Sci. USA* 86:7311-7315, 1989.

The biological activities of zveg3 antagonists can be studied in non-human animals by administration of exogenous compounds, by expression of zveg3 antisense polynucleotides, and by suppression of endogenous zveg3 expression through knock-out techniques. Viral delivery systems (disclosed above) can be

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employed. Zveg3 antagonists can be administered or expressed individually, in combination with other zveg3 antagonists, or in combination other compounds, including other growth factor antagonists. Test animals are monitored for changes in such parameters as clinical signs, body weight, blood cell counts, clinical chemistry, histopathology, and the like.

Effects of zveg3 antagonists on liver and kidney fibrosis can be tested in known animal models, such as the db/db mouse model disclosed by Cohen et al., *Diabetologia* 39:270-274, 1996 and Cohen et al., *J. Clin. Invest.* 95:2338-2345, 1995, transgenic animal models (Imai et al., *Contrib. Nephrol.* 107:205-215, 1994), and the CCl<sub>4</sub>-induced cirrhosis model (Rojkind and Greenwel, *Adv. Vet. Sci. Comp. Med.* 37:333-355, 1993; Díaz-Gil et al., *J. Hepatol.* 30:1065-1072, 1999).

Effects on lung fibrosis can be assayed in a mouse model using bleomycin. The chemotherapy agent bleomycin is a known causative agent of pulmonary fibrosis in humans and can induce interstitial lung disease in mice, including an increase in the number of fibroblasts, enhanced collagen deposition, and dysregulated matrix remodeling. C57Bl/6 mice are administered bleomycin by osmotic minipump for 1 week. There follows a period of inflammation, with cutaneous toxicity beginning approximately 4-7 days after bleomycin administration and continuing for about a week, after which the mice appear to regain health. About 3-4 weeks after the finish of bleomycin delivery, the mice are sacrificed, and the lungs are examined histologically for signs of fibrosis. Scoring is based on the extent of lung fibrotic lesions and their severity. Serum is assayed for lactic dehydrogenase, an intracellular enzyme that is released into the circulation upon general cell death or injury. Lung tissue is assayed for hydroxyproline as a measure of collagen deposition.

The invention is further illustrated by the following non-limiting examples.

## EXAMPLES

### Example 1

A human salivary gland library was screened for a full-length clone of zveg3 by PCR. This library was an arrayed library representing  $9.6 \times 10^5$  clones made in the vector pZP5x. The vector pZP5x is the same as vector pZP-9 (deposited with American Type Culture Collection, 10801 University Blvd., Manassas, VA under Accession Number 98668), but contains a cytomegalovirus promoter instead of a metallothionein promoter between the Asp718 and BamHI sites. The plasmid thus comprises a dihydrofolate reductase gene under control of the SV40 early promoter and SV40 polyadenylation site, and a cloning site to insert the gene of interest under control

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of the CMV promoter and the human growth hormone (hGH) gene polyadenylation site. The working plate containing 80 pools of 12,000 colonies each was screened by PCR using oligonucleotide primers ZC19,045 (SEQ ID NO:6) and ZC19,047 (SEQ ID NO:7) with an annealing temperature of 60°C for 35 cycles. There were two strong positives, pools 58 (T-8 F1-F12) and 77 (T-7 H1-H12). The corresponding pools in the transfer plate were then screened by PCR using the same conditions. Two positives were obtained at the transfer level. The positives were T-7 H11 and T-8 F10. 5' RACE reactions were done on the transfer plate pools, and the fragments were sequenced to check zveg3 sequence and determine if a full-length clone was present. For PCR, oligonucleotide primers ZC12,700 (SEQ ID NO:8) and ZC19,045 (SEQ ID NO:6) were used at an annealing temperature of 61°C for 5 cycles, then 55°C for 30 cycles. Sequencing showed that the pool T-7 H11 had a frameshift. Transfer plate 8 pool F10 sequence appeared to be correct, so this pool of DNA was used in filter lifts.

Pool F10 from transfer plate 8 was plated and filter lifted using nylon membranes (Hybond-N™; Amersham Corporation). Approximately 1200 colonies per plate on each of 5 filters were lifted for a total of approximately 6000 colonies. The filters were marked with a hot needle for orientation, then denatured for 6 minutes in 0.5 M NaOH and 1.5 M Tris-HCl, pH 7.2. The filters were then neutralized in 1.5 M NaCl and 0.5 M Tris-HCl, pH 7.2 for 6 minutes. The DNA was affixed to the filters using a UV crosslinker (Stratalinker®, Stratagene, La Jolla, CA) at 1200 joules. The filters were prewashed at 65°C in prewash buffer consisting of 0.25X SSC, 0.25% SDS, and 1mM EDTA. The solution was changed a total of three times over a 45-minute period to remove cell debris. Filters were prehybridized for approximately 3 hours at 65°C in 25 ml of ExpressHyb™. The probe was generated using an approximately 400-bp fragment produced by digestion of the first proprietary database clone with EcoRI and BglII and gel-purified using a spin column as disclosed above. The probe was radioactively labeled with <sup>32</sup>P by random priming as disclosed above and purified using a push column. ExpressHyb™ solution was used for the hybridizing solution for the filters. Hybridization took place overnight at 65°C. Blots were rinsed 2X in 65°C solution 1 (2X SSC, 0.1% SDS), then washed 4 times in solution 1 at 65°C. The filters were exposed to film overnight at -80°C. There were 14 positives on the filters. 85 clones were picked from the positive areas and screened by PCR using oligonucleotide primers ZC19,045 (SEQ ID NO:6) and ZC19,047 (SEQ ID NO:7) and an annealing temperature of 60°C. Thirteen positives were obtained and streaked out for individual clones. Twenty-four colonies were picked and checked by PCR as previously described. Six positives were obtained, two of which were sequenced. Both sequences were the same and full length. The sequence is shown in SEQ ID NO:1.

Example 2

A PCR panel was screened for mouse zveg3 DNA. The panel contained 8 cDNA samples from brain, bone marrow, 15-day embryo, testis, salivary gland, placenta, 15-day embryo (Clontech Laboratories), and 17-day embryo (Clontech Laboratories) libraries.

PCR mixtures contained oligonucleotide primers ZC21,222 (SEQ ID NO:9) and ZC21,224 (SEQ ID NO:10). The reaction was run at an annealing temperature of 66°C with an extension time of 2 minutes for a total of 35 cycles using Ex Taq™ DNA polymerase (PanVera, Madison, WI) plus antibody. DNA samples found to be positive for zveg3 by PCR and confirmed by sequencing included mouse 15-day embryo library total pool cDNA, mouse 15-day embryo (Clontech Laboratories) and 17-day embryo (both obtained from Clontech Laboratories), mouse salivary gland library total pool cDNA, and mouse testis library total pool cDNA. Fragments of about 600 bp from each of the mouse 15-day embryo library total pool cDNA, mouse 15-day embryo mcDNA, and mouse 17-day embryo mcDNA PCR products were sequenced. Sequence from the mouse 17-day embryo mcDNA and mouse 15-day embryo library total pool cDNA products confirmed the fragments to be mouse zveg3 DNA.

The mouse 15-day embryo library was screened for full-length zveg3 DNA. This library was an arrayed library representing  $9.6 \times 10^5$  clones in the pCMV•SPORT 2 vector (Life Technologies, Gaithersburg, MD). The working plate, containing 80 pools of 12,000 colonies each, was screened by PCR using oligonucleotide primers ZC21,223 (SEQ ID NO:11) and ZC21,224 (SEQ ID NO:10) with an annealing temperature of 66°C for 35 cycles. Eighteen positives were obtained. Fragments from four pools (A2, A10, B2, and C4) were sequenced; all were confirmed to encode zveg3. Additional rounds of screening using the same reaction conditions and pools from the working and source plates identified one positive pool (5D).

Positive colonies were screened by hybridization. Pool 5D from original source plate #5 was plated at about 250 colonies per plate and transferred to nylon membranes (Hybond-N™; Amersham Corporation, Arlington Heights, IL). Five filters were lifted for a total of ~1250 colonies. The filters were marked with a hot needle for orientation, then denatured for 6 minutes in 0.5 M NaOH and 1.5 M Tris-HCl, pH 7.2. The filters were then neutralized in 1.5 M NaCl and 0.5 M Tris-HCl, pH 7.2 for 6 minutes. The DNA was fixed to the filters using a UV crosslinker (Stratalinker®, Stratagene, La Jolla, CA) at 1200 joules. A probe was generated by PCR using oligonucleotide primers ZC21,223 (SEQ ID NO:11) and ZC21,224 (SEQ ID NO:10), and a mouse 15-day embryo template at an annealing temperature of 66°C for 35

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cycles. The PCR fragment was gel purified using a spin column containing a silica gel membrane (QIAquick™ Gel Extraction Kit; Qiagen, Inc., Valencia, CA). The DNA was radioactively labeled with <sup>32</sup>P using a commercially available kit (Rediprime™ II random-prime labeling system; Amersham Corp., Arlington Heights, IL) according to the manufacturer's specifications. The probe was purified using a commercially available push column (NucTrap® column; Stratagene, La Jolla, CA; see U.S. Patent No. 5,336,412). The filters were prewashed at 65°C in prewash buffer consisting of 0.25X SSC, 0.25% SDS and 1mM EDTA. The solution was changed a total of three times over a 45-minute period to remove cell debris. Filters were prehybridized overnight at 65°C in 25 ml of a hybridization solution (ExpressHyb™ Hybridization Solution; Clontech Laboratories, Inc., Palo Alto, CA), then hybridized overnight at 65°C in the same solution. Filters were rinsed twice at 65°C in pre-wash buffer (0.25X SSC, 0.25% SDS, and 1mM EDTA), then washed twice in pre-wash buffer at 65°C. Filters were exposed to film for 2 days at -80°C. There were 10 positives on the filters. 3 clones were picked from the positive areas, streaked out, and 15 individual colonies from these three positives were screened by PCR using primers ZC21,223 (SEQ ID NO:11) and ZC21,334 (SEQ ID NO:12) at an annealing temp of 66°C. Two positives were recovered and sequenced. Both sequences were found to be the same and encoded full-length mouse zveg3 (SEQ ID NO:4).

The amino acid sequence is highly conserved between mouse and human zveg3s, with an overall amino acid sequence identity of 87%. The secretory peptide, CUB domain, inter-domain, and growth factor domain have 82%, 92%, 79% and 94% amino acid identity, respectively.

### Example 3

A mammalian cell expression vector for the growth factor domain of zveg3 was constructed by joining the zveg3 fragment to a sequence encoding an optimized t-PA secretory signal sequence (U.S. Patent No. 5,641,655) in the linearized pZMP11 vector downstream of the CMV promoter. The plasmid pZMP11 is a mammalian expression vector containing an expression cassette having the CMV immediate early promoter, a consensus intron from the variable region of mouse immunoglobulin heavy chain locus, Kozak sequences, multiple restriction sites for insertion of coding sequences, a stop codon, and a human growth hormone terminator. The plasmid also contains an IRES element from poliovirus, the extracellular domain of CD8 truncated at the C-terminal end of the transmembrane domain, an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene, the SV40 terminator, and



the URA3 and CEN-ARS sequences required for selection and replication in *S. cerevisiae*. The resulting vector was designated pZMP11/zv3GF-otPA.

BHK 570 cells were transfected with pZMP11/zv3GF-otPA by liposome-mediated transfection (using Lipofectamine™; Life Technologies) and  
5 cultured according to conventional procedures.

BHK cell-conditioned media was adjusted to 20 mM MES at pH 5.5. A column of cation exchange resin (Poros® HS 50; PerSeptive Biosystems, Framingham, MA) (2-cm diameter; 50 ml bed volume) was equilibrated in 20 mM MES, 150 mM NaCl, pH 5.5. The adjusted media was pumped into the column at 20 ml/minute.  
10 When loading was completed, the column was washed, first in 20 mM MES, 150 mM NaCl, pH 5.5, then with the same composition buffer at pH 6.0. Once the baseline was back to zero absorbance, the column was eluted with a 10 column volume gradient to 20mM MES, 1M NaCl, pH 6.0. The zveg3 growth factor domain eluted at 25 to 50 mS conductivity during the evolving gradient. Reducing SDS-PAGE revealed a  
15 distinct band at 20 kD, which was confirmed as zveg3 by Western blotting. This material was pooled and prepared for loading to a tandem column array comprising a strong anion exchange resin (Poros® HQ 50; PerSeptive Biosystems) followed by an immobilized metal (nickel) affinity column in series. The system of columns was equilibrated in 20 mM MOPS buffer at pH 7.0. The vegf3 pool was in-line diluted at  
20 1:10 (V:V) with the MOPS equilibration buffer while loading. After loading was completed the column series was washed with 20 mM MOPS pH 7.0 buffer until baseline absorbance was obtained. The nickel column was then disconnected from the anion exchanger and washed with 20 mM MOPS pH 7.0 containing 150 mM NaCl. The column was then eluted with a 1 column volume gradient between the last washing  
25 buffer and the same buffer containing 20 mM imidazole at pH 7.0. The fractions containing the zveg3 growth factor domain were pooled and concentrated using 5 kD cutoff membrane in preparation for buffer exchange and polishing on a size exclusion column equilibrated in PBS.

#### 30 Example 4

To make transgenic animals expressing zveg3 genes requires adult, fertile males (studs) (B6C3f1, 2-8 months of age (Taconic Farms, Germantown, NY)), vasectomized males (duds) (B6D2f1, 2-8 months, (Taconic Farms)), prepubescent fertile females (donors) (B6C3f1, 4-5 weeks, (Taconic Farms)) and adult fertile females  
35 (recipients) (B6D2f1, 2-4 months, (Taconic Farms)).

The donors are acclimated for 1 week, then injected with approximately 8 IU/mouse of Pregnant Mare's Serum gonadotrophin (Sigma, St. Louis, MO) I.P., and

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46-47 hours later, 8 IU/mouse of human Chorionic Gonadotropin (hCG (Sigma)) I.P. to induce superovulation. Donors are mated with studs subsequent to hormone injections. Ovulation generally occurs within 13 hours of hCG injection. Copulation is confirmed by the presence of a vaginal plug the morning following mating.

- 5 Fertilized eggs are collected under a surgical scope (Leica MZ12 Stereo Microscope, Leica, Wetzlar, Germany). The oviducts are collected and eggs are released into urinalysis slides containing hyaluronidase (Sigma Chemical Co.). Eggs are washed once in hyaluronidase, and twice in Whitten's W640 medium (Table 2; all reagents available from Sigma Chemical Co.) that has been incubated with 5% CO<sub>2</sub>,  
10 5% O<sub>2</sub>, and 90% N<sub>2</sub> at 37°C. The eggs are stored in a 37°C/5% CO<sub>2</sub> incubator until microinjection.

Table 2

	<u>mgs/200 ml</u>	<u>mgs/500 ml</u>
NaCl	1280	3200
KCl	72	180
KH <sub>2</sub> PO <sub>4</sub>	32	80
MgSO <sub>4</sub> · 7H <sub>2</sub> O	60	150
Glucose	200	500
Ca <sup>2+</sup> Lactate	106	265
Benzylpenicillin	15	37.5
Streptomycin SO <sub>4</sub>	10	25
NaHCO <sub>3</sub>	380	950
Na Pyruvate	5	12.5
H <sub>2</sub> O	200 ml	500 ml
500 mM EDTA	100 µl	250 µl
5% Phenol Red	200 µl	500 µl
BSA	600	1500

- 15 Zveg3 cDNA is inserted into the expression vector pHB12-8. Vector pHB12-8 was derived from p2999B4 (Palmiter et al., *Mol. Cell Biol.* 13:5266-5275, 1993) by insertion of a rat insulin II intron (ca. 200 bp) and polylinker (Fse I/Pme I/Asc I) into the Nru I site. The vector comprises a mouse metallothionein (MT-1) promoter (ca. 750 bp) and human growth hormone (hGH) untranslated region and

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polyadenylation signal (ca. 650 bp) flanked by 10 kb of MT-1 5' flanking sequence and 7 kb of MT-1 3' flanking sequence. The cDNA is inserted between the insulin II and hGH sequences.

10-20 micrograms of plasmid DNA is linearized, gel-purified, and resuspended in 10 mM Tris pH 7.4, 0.25 mM EDTA pH 8.0, at a final concentration of 5-10 nanograms per microliter for microinjection.

Plasmid DNA is microinjected into harvested eggs contained in a drop of W640 medium overlaid by warm, CO<sub>2</sub>-equilibrated mineral oil. The DNA is drawn into an injection needle (pulled from a 0.75mm ID, 1mm OD borosilicate glass capillary) and injected into individual eggs. Each egg is penetrated with the injection needle into one or both of the haploid pronuclei.

Picoliters of DNA are injected into the pronuclei, and the injection needle is withdrawn without coming into contact with the nucleoli. The procedure is repeated until all the eggs are injected. Successfully microinjected eggs are transferred into an organ tissue-culture dish with pregassed W640 medium for storage overnight in a 37°C/5% CO<sub>2</sub> incubator.

The following day, 2-cell embryos are transferred into pseudopregnant recipients. The recipients are identified by the presence of copulation plugs, after copulating with vasectomized duds. Recipients are anesthetized and shaved on the dorsal left side and transferred to a surgical microscope. A small incision is made in the skin and through the muscle wall in the middle of the abdominal area outlined by the ribcage, the saddle, and the hind leg, midway between knee and spleen. The reproductive organs are exteriorized onto a small surgical drape. The fat pad is stretched out over the surgical drape, and a baby serrefine (Roboz, Rockville, MD) is attached to the fat pad and left hanging over the back of the mouse, preventing the organs from sliding back in.

With a fine transfer pipette containing mineral oil followed by alternating W640 and air bubbles, 12-17 healthy 2-cell embryos from the previous day's injection are transferred into the recipient. The swollen ampulla is located and holding the oviduct between the ampulla and the bursa, and a nick in the oviduct is made with a 28 g needle close to the bursa, making sure not to tear the ampulla or the bursa.

The pipette is transferred into the nick in the oviduct, and the embryos are blown in, allowing the first air bubble to escape the pipette. The fat pad is gently pushed into the peritoneum, and the reproductive organs are allowed to slide in. The peritoneal wall is closed with one suture, and the skin is closed with a wound clip. The mice recuperate on a 37°C slide warmer for a minimum of 4 hours.

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The recipients are returned to cages in pairs, and allowed 19-21 days gestation. After birth, 19-21 days postpartum is allowed before weaning. The weanlings are sexed and placed into separate sex cages, and a 0.5 cm biopsy (used for genotyping) is snipped off the tail with clean scissors.

5 Genomic DNA is prepared from the tail snips using a commercially available kit (DNeasy™ 96 Tissue Kit; Qiagen, Valencia, CA) following the manufacturer's instructions. Genomic DNA is analyzed by PCR using primers designed to the human growth hormone (hGH) 3' UTR portion of the transgenic vector. The use of a region unique to the human sequence (identified from an alignment of the  
10 human and mouse growth hormone 3' UTR DNA sequences) ensures that the PCR reaction does not amplify the mouse sequence. Primers ZC17,251 (SEQ ID NO:13) and ZC17,252 (SEQ ID NO:14) amplify a 368-base-pair fragment of hGH. In addition, primers ZC17,156 (SEQ ID NO:15) and ZC17,157 (SEQ ID NO:16), which hybridize to vector sequences and amplify the cDNA insert, may be used along with the hGH  
15 primers. In these experiments, DNA from animals positive for the transgene will generate two bands, a 368-base-pair band corresponding to the hGH 3' UTR fragment and a band of variable size corresponding to the cDNA insert.

Once animals are confirmed to be transgenic (TG), they are back-crossed into an inbred strain by placing a TG female with a wild-type male, or a TG  
20 male with one or two wild-type female(s). As pups are born and weaned, the sexes are separated, and their tails snipped for genotyping.

To check for expression of a transgene in a live animal, a partial hepatectomy is performed. A surgical prep is made of the upper abdomen directly below the xiphoid process. Using sterile technique, a small 1.5-2 cm incision is made  
25 below the sternum, and the left lateral lobe of the liver is exteriorized. Using 4-0 silk, a tie is made around the lower lobe securing it outside the body cavity. An atraumatic clamp is used to hold the tie while a second loop of absorbable Dexon (American Cyanamid, Wayne, N.J.) is placed proximal to the first tie. A distal cut is made from the Dexon tie, and approximately 100 mg of the excised liver tissue is placed in a  
30 sterile petri dish. The excised liver section is transferred to a 14-ml polypropylene round bottom tube, snap frozen in liquid nitrogen, and stored on dry ice. The surgical site is closed with suture and wound clips, and the animal's cage is placed on a 37°C heating pad for 24 hours post-operatively. The animal is checked daily post-operatively, and the wound clips are removed 7-10 days after surgery.

35 Analysis of the mRNA expression level of each transgene is done using an RNA solution hybridization assay or real-time PCR on an ABI Prism 7700 (PE Applied Biosystems, Inc., Foster City, CA) following the manufacturer's instructions.

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An adenovirus vector was prepared using a liver-specific albumin gene enhancer and basal promoter (designated "AEO promoter"). The albumin promoter construct (designated pAEO) was constructed by inserting a 2.2 kb NotI/EcoRV fragment from pALBdelta2L (Pinkert et al., *Genes Dev.* 1:268-276, 1987) and an 850 bp NruI/NotI DNA segment comprising the rat insulin II intron, an FseI/PmeI/AscI polylinker, and the human growth hormone poly A sequence into a commercially available phagemid vector (pBluescript® KS(+); Stratagene, La Jolla, CA). For microinjection, the plasmid is digested with NotI to liberate the expression cassette.

An additional adenovirus vector was constructed using an epithelial cell-specific keratin gene (K14) promoter (Vassar et al., *Proc. Natl. Acad. Sci. USA* 86:1563-1567, 1989). The 1038-bp open reading frame encoding full-length human zveg3 was amplified by PCR so as to introduce an optimized initiation codon and flanking 5' PmeI and 3' AscI sites using the primers ZC20,180 (SEQ ID NO:17) and ZC20,181 (SEQ ID NO:18). The resulting PmeI/AscI fragment was subcloned into the polylinker of pKFO114, a basal keratinocyte-restricted transgenic vector comprising the human keratin 14 (K14) promoter (an approximately 2.3 Kb fragment amplified from human genomic DNA [obtained from Clontech Laboratories, Inc.] based on the sequence of Staggers et al., "Sequence of the promoter for the epidermal keratin gene, K14", GenBank accession #U11076, 1994), followed by a heterologous intron (a 294-bp BstXI/PstI fragment from pIRES1hyg (Clontech Laboratories, Inc.; see, Huang and Gorman, *Nucleic Acids Res.* 18:937-947, 1990), a PmeI/AscI polylinker, and the human growth hormone gene polyadenylation signal (a 627 bp SmaI/EcoRI fragment; see, Seeburg, *DNA* 1:239-249, 1982). The transgene insert was separated from the plasmid backbone by NotI digestion and agarose gel purification, and fertilized ova from matings of B6C3F1Tac mice or inbred FVB/NTac mice were microinjected and implanted into pseudopregnant females essentially as described by Malik et al., *Molec. Cell. Biol.* 15:2349-2358, 1995. Transgenic founders were identified by PCR on genomic tail DNA using primers specific for the human growth hormone poly A signal (ZC17,252, SEQ ID NO:14; and ZC17,251, SEQ ID NO:13) to amplify a 368-bp diagnostic product. Transgenic lines were initiated by breeding founders with C57BL/6Tac or FVB/NTac mice.

Transgenic mice were generated essentially as disclosed above using MT-1, K14, and AEO promoters. Four MT-1/zveg3 transgenic mice were generated. In one animal (female) approximately 800 molecules zveg3 mRNA/cell were produced in the liver after zinc induction. This animal had enlargement of the liver and spleen. Also observed were proliferation of hepatic sinusoidal cells and extra-medullary hematopoiesis. One K14/zveg3 transgenic mouse (female) showed a low level of

expression with low body weight, low hematocrit, and low platelet count. One AEO/zveg3 transgenic mouse (male) with a low level of expression exhibited liver sinusoidal cell proliferation.

Histological analysis was carried out on livers from N1 transgenic mice overexpressing full-length zveg3 from the AEO promoter and nontransgenic littermate controls at 8, 16, 22, and 33 weeks of age. Similar changes were seen in male and female animals. H & E and trichrome stains indicate a definite increase in number of liver sinusoidal (stellate) cells and increased perisinusoidal extracellular matrix (EMC) deposition at 8 weeks of age. There was a persistent increase in number of stellate cells and in the amount and thickness of perisinusoidal EMC as well as some perivenular EMC deposition by 16 weeks. At 22 weeks similar changes were seen, but with an increase in incidence and severity of perivenular fibrosis (steato fibrosis). Changes similar to those at 16 and 22 weeks were observed at 33 weeks, however some animals had fibrotic banding and multiple, encapsulated areas in which hepatocytes appeared enlarged and vacuolated. These areas tended to be surrounded by a fibrous tissue capsule, and the hepatocytes within these areas appeared normal. These changes were consistent with early cirrhosis.

Similar changes were seen in 8-week-old N2 mice and in breeder males sacrificed at approximately 32-34 weeks.

#### Example 5

For construction of adenovirus vectors, the protein coding region of human zveg3 was amplified by PCR using primers that added PmeI and AscI restriction sites at the 5' and 3' termini respectively. PCR primers ZC20,180 (SEQ ID NO:17) and ZC20,181 (SEQ ID NO:18) were used with a full-length zveg3 cDNA template in a PCR reaction as follows: incubation at 95°C for 5 minutes; followed by 15 cycles at 95°C for 1 min., 61°C for 1 min., and 72°C for 1.5 min.; followed by 72°C for 7 min.; followed by a 4°C soak. The reaction product was loaded onto a 1.2 % low-melting-temperature agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The zveg3 PCR product was excised from the gel and purified using a commercially available kit comprising a silica gel membrane spin column (QIAquick™ PCR Purification Kit and gel cleanup kit; Qiagen, Inc.) as per kit instructions. The zveg3 product was then digested with PmeI and AscI, phenol/chloroform extracted, EtOH precipitated, and rehydrated in 20 ml TE (Tris/EDTA pH 8). The 1038 bp zveg3 fragment was then ligated into the PmeI-AscI sites of the transgenic vector pTG12-8 (also known as pHB12-8; see Example 4) and transformed into *E. coli* DH10B™

competent cells by electroporation. Clones containing zveg3 were identified by plasmid DNA miniprep followed by digestion with PmeI and AscI. A positive clone was sequenced to insure that there were no deletions or other anomalies in the construct. The sequence of zveg3 cDNA was confirmed.

5 DNA was prepared using a commercially available kit (Maxi Kit, Qiagen, Inc.), and the 1038bp zveg3 cDNA was released from the pTG12-8 vector using PmeI and AscI enzymes. The cDNA was isolated on a 1% low melting temperature agarose gel and was excised from the gel. The gel slice was melted at 70°C, and the DNA was extracted twice with an equal volume of Tris-buffered phenol  
10 and precipitated with EtOH. The DNA was resuspended in 10 µl H<sub>2</sub>O.

The zveg3 cDNA was cloned into the EcoRV-AscI sites of a modified pAdTrack-CMV (He, T-C. et al., *Proc. Natl. Acad. Sci. USA* 95:2509-2514, 1998). This construct contains the green fluorescent protein (GFP) marker gene. The CMV promoter driving GFP expression was replaced with the SV40 promoter, and the SV40  
15 polyadenylation signal was replaced with the human growth hormone polyadenylation signal. In addition, the native polylinker was replaced with FseI, EcoRV, and AscI sites. This modified form of pAdTrack-CMV was named pZyTrack. Ligation was performed using a commercially available DNA ligation and screening kit (Fast-Link™ kit; Epicentre Technologies, Madison, WI). Clones containing zveg3 were identified  
20 by digestion of mini prep DNA with FseI and AscI. In order to linearize the plasmid, approximately 5 µg of the resulting pZyTrack zveg3 plasmid was digested with PmeI. Approximately 1 µg of the linearized plasmid was cotransformed with 200 ng of supercoiled pAdEasy (He et al., *ibid.*) into *E. coli* BJ5183 cells (He et al., *ibid.*). The co-transformation was done using a Bio-Rad Gene Pulser at 2.5 kV, 200 ohms and 25  
25 µFa. The entire co-transformation mixture was plated on 4 LB plates containing 25 µg/ml kanamycin. The smallest colonies were picked and expanded in LB/kanamycin, and recombinant adenovirus DNA was identified by standard DNA miniprep procedures. Digestion of the recombinant adenovirus DNA with FseI and AscI confirmed the presence of the zveg3 insert. The recombinant adenovirus miniprep  
30 DNA was transformed into *E. coli* DH10B™ competent cells, and DNA was prepared using a Maxi Kit (Qiagen, Inc.) according to kit instructions.

Approximately 5 µg of recombinant adenoviral DNA was digested with PacI enzyme (New England Biolabs) for 3 hours at 37°C in a reaction volume of 100 µl containing 20-30U of PacI. The digested DNA was extracted twice with an equal  
35 volume of phenol/chloroform and precipitated with ethanol. The DNA pellet was resuspended in 10µl distilled water. A T25 flask of QBI-293A cells (Quantum Biotechnologies, Inc. Montreal, Qc. Canada), inoculated the day before and grown to

60-70% confluence, were transfected with the PacI digested DNA. The PacI-digested DNA was diluted up to a total volume of 50  $\mu$ l with sterile HBS (150mM NaCl, 20mM HEPES). In a separate tube, 20  $\mu$ l of 1mg/ml N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium salts (DOTAP) (Boehringer Mannheim, Indianapolis, IN) was  
 5 diluted to a total volume of 100  $\mu$ l with HBS. The DNA was added to the DOTAP, mixed gently by pipeting up and down, and left at room temperature for 15 minutes. The media was removed from the 293A cells and washed with 5 ml serum-free minimum essential medium (MEM) alpha containing 1mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, and 25mM HEPES buffer (reagents obtained from  
 10 Life Technologies, Gaithersburg, MD). 5 ml of serum-free MEM was added, and the cells were held at 37°C. The DNA/lipid mixture was added drop-wise to the flask of cells, mixed gently, and incubated at 37°C for 4 hours. The media containing the DNA/lipid mixture was then aspirated off and replaced with 5 ml complete MEM containing 5% fetal bovine serum. The transfected cells were monitored for GFP  
 15 expression and formation of foci (viral plaques).

Seven days after transfection of 293A cells with the recombinant adenoviral DNA, the cells expressed GFP and started to form foci. The crude viral lysate was collected using a cell scraper to *collect* all of the 293A cells. The lysate was transferred to a 50-ml conical tube. To release most of the virus particles from the  
 20 cells, three freeze/thaw cycles were done in a dry ice/ethanol bath and a 37° waterbath.

The crude lysate was amplified (Primary (1°) amplification) to obtain a working stock of zvegf3 rAdV lysate. Ten 10-cm plates of nearly confluent (80-90%) 293A cells were set up 20 hours *previously*, 200  $\mu$ l of crude rAdV lysate added to each 10-cm plate and monitored for 48 to 72 hours looking for CPE under the white light  
 25 microscope and expression of GFP under the fluorescent microscope. When all of the cells showed CPE (Cytopathic Effect) this 1° stock lysate was collected and freeze/thaw cycles performed as described above.

Secondary (2°) amplification of zvegf3 rAdV was obtained as follows: Twenty 15-cm tissue culture dishes of 293A cells were prepared so that the cells were  
 30 80-90% confluent. All but 20 ml of 5% MEM media was removed, and each dish was inoculated with 300-500  $\mu$ l of the 1° amplified rAdv lysate. After 48 hours the cells were lysed from virus production, the lysate was collected into 250-ml polypropylene centrifuge bottles, and the rAdV was purified.

NP-40 detergent was added to a final concentration of 0.5% to the  
 35 bottles of crude lysate in order to lyse all cells. Bottles were placed on a rotating platform for 10 minutes agitating as fast as possible without the bottles falling over. The debris was pelleted by centrifugation at 20,000 X G for 15 minutes. The



supernatant was transferred to 250-ml polycarbonate centrifuge bottles, and 0.5 volume of 20% PEG8000/2.5 M NaCl solution was added. The bottles were shaken overnight on ice. The bottles were centrifuged at 20,000 X G for 15 minutes and, the supernatant was discarded into a bleach solution. Using a sterile cell scraper, the white, virus/PEG precipitate from 2 bottles was resuspended in 2.5 ml PBS. The resulting virus solution was placed in 2-ml microcentrifuge tubes and centrifuged at 14,000 X G in the microcentrifuge for 10 minutes to remove any additional cell debris. The supernatant from the 2-ml microcentrifuge tubes was transferred into a 15-ml polypropylene snapcap tube and adjusted to a density of 1.34 g/ml with CsCl. The volume of the virus solution was estimated, and 0.55 g/ml of CsCl was added. The CsCl was dissolved, and 1 ml of this solution weighed 1.34 g. The solution was transferred to 3.2-ml, polycarbonate, thick-walled centrifuge tubes and spun at 348,000 X G for 3-4 hours at 25°C. The virus formed a white band. Using wide-bore pipette tips, the virus band was collected.

The virus recovered from the gradient had a large amount of CsCl which had to be removed before it was used on cells. Commercially available ion-exchange columns (PD-10 columns prepacked with Sephadex® G-25M; Pharmacia Biotech, Piscataway, NJ) were used to desalt the virus preparation. The column was equilibrated with 20 ml of PBS. The virus was loaded and allowed to run into the column. 5 ml of PBS was added to the column, and fractions of 8-10 drops were collected. The optical density of a 1:50 dilution of each fraction was determined at 260 nm on a spectrophotometer. A clear absorbance peak was present between fractions 7-12. Peak fractions were pooled, and the optical density (OD) of a 1:25 dilution was determined. OD was converted to virus concentration using the formula: (OD at 260nm)(25)( $1.1 \times 10^{12}$ ) = virions/ml. The OD of a 1:25 dilution of the zvegf3 rAdV was 0.145, giving a virus concentration of  $4 \times 10^{12}$  virions/ml.

To store the virus, glycerol was added to the purified virus to a final concentration of 15%, mixed gently but effectively, and stored in aliquots at -80°C.

A protocol developed by Quantum Biotechnologies, Inc. (Montreal, Canada) was followed to measure recombinant virus infectivity. Briefly, two 96-well tissue culture plates were seeded with  $1 \times 10^4$  293A cells per well in MEM containing 2% fetal bovine serum for each recombinant virus to be assayed. After 24 hours 10-fold dilutions of each virus from  $1 \times 10^{-2}$  to  $1 \times 10^{-14}$  were made in MEM containing 2% fetal bovine serum. 100 µl of each dilution was placed in each of 20 wells. After 5 days at 37°C, wells were read either positive or negative for Cytopathic Effect (CPE), and a value for plaque forming units (pfu)/ml was calculated.

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TCID<sub>50</sub> formulation used was as per Quantum Biotechnologies, Inc., above. The titer (T) was determined from a plate where virus was diluted from 10<sup>-2</sup> to 10<sup>-14</sup>, and read 5 days after the infection. At each dilution a ratio (R) of positive wells for CPE per the total number of wells was determined.

To calculate titer of the undiluted virus sample: the factor, "F" = 1+d(S-0.5); where "S" is the sum of the ratios (R); and "d" is Log10 of the dilution series, for example, "d" is equal to 1 for a ten-fold dilution series. The titer of the undiluted sample is  $T = 10^{(1+F)} = \text{TCID}_{50}/\text{ml}$ . To convert TCID<sub>50</sub>/ml to pfu/ml, 0.7 is subtracted from the exponent in the calculation for titer (T).

The zvegf3 adenovirus had a titer of  $1.8 \times 10^{10}$  pfu/ml.

#### Example 6

Treatment of mice with zvegf3-adenovirus led to changes in liver and spleen. The livers were pale and very enlarged, with enlarged vessels at the tips of the lobes. The livers also showed sinusoidal cell proliferation. Changes were also seen in hepatocytes (hypertrophy, degeneration, and necrosis) and were most likely non-specific effects of adenovirus infection. Splenic change consisted of increased extramedullary hematopoiesis, which was correlated with enlarged splenic size.

#### Example 7

Polyclonal anti-peptide antibodies were prepared by immunizing two female New Zealand white rabbits with the peptides huzvegf3-1 (residues 80-104 of SEQ ID NO:2), huzvegf3-2 (residues 299-314 of SEQ ID NO:2), huzvegf3-3 (residues 299-326 of SEQ ID NO:2 with an N-terminal cys residue), or huzvegf3-4 (residues 195-225 of SEQ ID NO:2 with a C-terminal cys residue). The peptides were synthesized using an Applied Biosystems Model 431A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions. The peptides huzvegf3-1, huzvegf3-3, and huzvegf3-4 were then conjugated to the carrier protein maleimide-activated keyhole limpet hemocyanin (KLH) through cysteine residues (Pierce Chemical Co., Rockford, IL). The peptide huzvegf3-2 was conjugated to the carrier protein KLH using glutaraldehyde. The rabbits were each given an initial intraperitoneal (IP) injection of 200 µg of conjugated peptide in Complete Freund's Adjuvant (Pierce Chemical Co.) followed by booster IP injections of 100 µg conjugated peptide in Incomplete Freund's Adjuvant every three weeks. Seven to ten days after the administration of the third booster injection, the animals were bled and the serum was collected. The rabbits were then boosted and bled every three weeks.

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The huzveg3 peptide-specific antibodies were affinity purified from the rabbit serum using an CNBr-Sepharose® 4B peptide column (Pharmacia Biotech) that was prepared using 10 mg of the respective peptides per gram CNBr-Sepharose®, followed by dialysis in PBS overnight. Peptide specific-huzveg3 antibodies were characterized by an ELISA titer check using 1 µg/ml of the appropriate peptide as an antibody target. The huzveg3-1 peptide-specific antibodies had a lower limit of detection (LLD) of 500 pg/ml by ELISA on the appropriate antibody target and recognize full-length recombinant protein (MBP-fusion) by ELISA. The huzveg3-2 peptide-specific antibodies had an LLD of 1 ng/ml by ELISA. The huzveg3-3 peptide-specific antibodies had an LLD of 50 pg/ml by ELISA and recognized recombinant protein by Western Blot analysis. The huzveg3-4 peptide-specific antibodies had an LLD of 50 pg/ml by ELISA and recognized recombinant protein by Western Blot analysis.

#### Example 8

Mouse hybridomas producing monoclonal antibodies (MAbs) specific for recombinant human zveg3 growth factor domain (GFD) protein were generated using purified, untagged, recombinant human zveg3 GFD produced in BHK cells (huzveg3-GFD-BHK). Ten BALB/c mice were each injected IP on day 1 with 20 µg of huzveg3-GFD-BHK mixed 1:1 (v/v) in complete Freund's adjuvant. Each mouse was subsequently injected IP with 10 µg of huzveg3-GFD-BHK mixed 1:1 in incomplete Freund's adjuvant on days 15, 29, 41, 57, 71, 89 and 115. On day 118, splenocytes and lymphocytes from enlarged lymph nodes of two mice with the highest anti-huzveg3 antibody titer (as determined in a biotinylated huzveg3-GFD capture ELISA; see below) were fused at a 2.76:1 ratio with the X63-Ag8.653 mouse myeloma cell line (Kearney et al., *J. Immunol.* **123**:1548-1550, 1979) essentially as disclosed by Lane (*J. Immunol. Methods* **81**:223-228, 1985). The fusion mixture was plated into 24 96-well plates at an average density of  $1.2 \times 10^5$  total cells/well in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal clone I serum (HyClone Laboratories, Inc., Logan, UT), 10% hybridoma cloning supplement (BM Condimed® H1; Roche Diagnostics Corp., Indianapolis, IN), 2 mM L-glutamine (Life Technologies, Inc.), 100 U/mL penicillin G sodium (Life Technologies, Inc.), and 100 µg/mL streptomycin sulfate (Life Technologies, Inc.). Wells were fed on days 4 and 7 by aspiration and replacement of approximately three-fourths of the media contents in each well. This fusion was designated HH1.

Anti-huzveg3 mAbs of the IgG class were detected on days 9/10 post-fusion using a biotinylated huveg3-GFD capture ELISA. Wells of plates (Immulon®

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II; Dynex Technologies, Chantilly, VA) were coated with 1  $\mu\text{g/mL}$  of goat anti-mouse IgG (obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 0.05 M carbonate/bicarbonate buffer (Sigma, St. Louis, MO), 50  $\mu\text{L/well}$ . Plates were incubated at 4°C overnight, then washed three times with phosphate buffered saline containing 0.05% polyoxyethylenesorbitan monolaurate (Tween 20) (PBST) to remove unbound antibody. Wells were blocked with PBST containing 1% (w/v) bovine serum albumin (Sigma) (PTB buffer), 200  $\mu\text{L/well}$ , for 1 hour at room temperature (RT), then washed as above. Culture supernatants from the fusion plates were replica-plated onto antibody-coated plates, 100  $\mu\text{L/well}$ , and incubated at RT for 1 hour. Human zveg3-GFD protein was biotinylated with sulfo-NHS-LC-biotin (Pierce Chemical Company, Rockford, IL) according to the manufacturer's instructions for 45 minutes at RT. The reaction was stopped with 2M glycine. Biotinylated protein was diluted to 1  $\mu\text{g/mL}$  in PTB buffer. The plates were washed four times with PBST, biotinylated huzveg3-GFD was added at 100  $\mu\text{L/well}$ , and the plates were incubated at RT for 1 hour. The plates were then washed four times with PBST, and HRP-conjugated streptavidin (Pierce Chemical Company) was diluted to 0.5  $\mu\text{g/mL}$  in PTB buffer and added 100 to the plates at  $\mu\text{L/well}$ . The plates were incubated at RT for 1 hour, then washed five times with PBST. TMB substrate (EIA chromagen, Cat. # R6R; Genetic Systems Corp.) was diluted 1:100 in substrate buffer (Genetic Systems Corp.) and added to the plates at 100  $\mu\text{L/well}$ . The plates were incubated 10-15 minutes at RT in the dark. Color development was stopped by the addition of 100  $\mu\text{L/well}$  of 1N  $\text{H}_2\text{SO}_4$ . Optical density was read on an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at wavelengths 450 nm (L1) and 650 nm (L2). Final OD measurement was determined by the formula L1-L2.

78 master wells contained antibody that was capable of capturing biotinylated huzveg3-GFD. These master wells were expanded for hybridoma cryopreservation and additional supernatant generation. ELISA analysis of the expanded master well supernatants demonstrated that 27 of the original 78 master wells retained antibody specific for huzveg3-GFD, with 20 of 27 possessing significant reactivity with the antigen.

Six of the designated master wells were cloned by limiting dilution at a density of less than one cell per well. Monoclonality was assessed by microscopic examination of wells for a single focus of cell growth. Clones were tested for specific antibody by the same assay used in the master screen. Five to six of the strongest clones were briefly expanded. Supernatants from these clones was then serially diluted and tested by ELISA to identify the three best antibody-producing clones. Upon

verification, the best clone from each master well was subsequently recloned and assayed as described above.

Six of the highest titered second round clones were adapted to growth in fusion/cloning medium minus the addition of hybridoma cloning supplement. Each of the clones was subsequently adapted to growth in a production medium formulation consisting essentially of Dulbecco's modified Eagle's medium + 2.5% fetal clone I serum and various supplements. Supernatants were again serially diluted and tested by ELISA on huzveg3-GFD to identify the highest titered and next highest titered clones (designated the primary and secondary clones, respectively). MAb produced by each set of clones was then evaluated for IgG subclass using the Mouse Hybridoma Subtyping Kit (Cat. #1183117; Roche Diagnostics Corp.). Clones HH1-24, -40, -57 and -76 were all found to produce an IgG<sub>1</sub> antibody. Clones HH1-58 and -78 produced an IgG<sub>2b</sub> antibody. All antibodies possessed a  $\kappa$  light chain.

#### 15 Example 9

Recombinant zveg3 was analyzed for mitogenic activity on rat stellate cells (Greenwel et al., *Laboratory Invest.* 65:644, 1991; Greenwel et al., *Laboratory Invest.* 69:210, 1993). Stellate cells were plated at a density of 2,000 cells/well in 96-well culture plates and grown for approximately 72 hours in DMEM containing 10% fetal calf serum at 37°C. Cells were quiesced by incubating them for 20 hours in serum-free DMEM/Ham's F-12 medium containing insulin (5  $\mu$ g/ml), transferrin (20  $\mu$ g/ml), and selenium (16 pg/ml) (ITS). At the time of the assay, the medium was removed, and test samples were added to the wells in triplicate. Test samples consisted of either conditioned media (CM) from adenovirally-infected HaCaT human keratinocyte cells (Boukamp et al., *J. Cell. Biol.* 106:761-771, 1988) expressing full-length zveg3, purified growth factor domain expressed in BHK cells, or control media from cells infected with parental adenovirus (Zpar) containing an expression unit for green fluorescent protein. The CM was concentrated 10-fold using a 15-ml centrifugal filter device with a 10K membrane filter (Ultrafree®; Millipore Corp., Bedford, MA), then diluted back to 3x with ITS medium and added to the cells. Purified protein in a buffer containing 0.1% BSA was serially diluted into ITS medium at concentrations of 1  $\mu$ g/ml to 1 ng/ml and added to the test plate. A control buffer of 0.1% BSA was diluted identically to the highest concentration of zveg3 protein and added to the plate. For measurement of [<sup>3</sup>H]thymidine incorporation, 20  $\mu$ l of a 50  $\mu$ Ci/ml stock in DMEM was added directly to the cells, for a final activity of 1  $\mu$ Ci/well. After another 24-hour incubation, mitogenic activity was assessed by measuring the uptake of [<sup>3</sup>H]thymidine. Media were removed, and cells were incubated with 0.1 ml of trypsin

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until cells detached. Cells were harvested onto 96-well filter plates using a sample harvester (FilterMate™ harvester; Packard Instrument Co., Meriden, CT). The plates were then dried at 65°C for 15 minutes, sealed after adding 40 µl/well scintillation cocktail (Microscint™ O; Packard Instrument Co.) and counted on a microplate scintillation counter (Topcount®; Packard Instrument Co.).

Results, presented in Table 3, demonstrated that zveg3 CM had approximately 4.4-fold higher mitogenic activity on stellate cells over control CM, and purified protein at 100 ng/ml caused a maximal 14-fold increase in [<sup>3</sup>H]thymidine incorporation over the buffer control.

Table 3

<u>Sample</u>	<u>CPM Incorporated</u>	
	<u>Mean</u>	<u>St. dev.</u>
zveg3 (2x CM)	42489	1306
Zpar (2x CM)	9629	540
zveg3 GF domain, 100 ng/ml	77540	4142
zveg3 GF domain, 33.3 ng/ml	74466	18142
zveg3 GF domain, 11.1 ng/ml	52462	6239
zveg3 GF domain, 3.7 ng/ml	15128	4989
Buffer control	5618	573
PDGF-BB 20 ng/ml	19741	2075
PDGF-AA 20 ng/ml	33133	3325
Media alone (basal response)	6765	226

Example 10

Northern blot analysis was performed on 2 µg samples of poly(A)<sup>+</sup> RNA from five mouse prostate cell lines (designated Jakotay, Nelix, Paris, Torres, and Tuvak). Total RNA from mouse and rat liver were used as controls (20 µg each). An approximately 680-bp DNA probe was generated by PCR using oligonucleotide primers ZC21,222 (SEQ ID NO:9) and ZC21,224 (SEQ ID NO:10) and a mouse zveg3 full-length cDNA clone as a template. The DNA probe was purified by conventional procedures using a commercially available kit (QIAquick™ Gel Extraction Kit; Qiagen, Inc., Valencia, CA). The probe was radioactively labeled with <sup>32</sup>P using a commercially available kit (Rediprime™ II DNA Labeling system; Amersham,

Arlington Heights, IL) according to the manufacturer's specifications. The probe was purified using a push column (NucTrap® column; Stratagene, La Jolla, CA; see U.S. Patent No. 5,336,412). A commercially available hybridization solution (ExpressHyb™ Hybridization Solution; Clontech Laboratories, Inc., Palo Alto, CA) was used for the blots. Hybridization took place overnight at 65°C. The blots were then washed four times in 2X SCC and 0.1% SDS at room temperature, followed by two washes in 0.1X SSC and 0.1% SDS at 55° C. One transcript size was detected at approximately 4 kb in Jakotay, Nelix, Paris and Tuvak cell lines. Signal intensity was highest for Jakotay, then Nelix and Tuvak.

#### Example 11

Recombinant human zveg3 GFD produced in BHK cells was assayed for growth factor activity using an osteoblast cell line containing a luciferase reporter gene construct. CCC4 cells (derived from p53 knock-out mice) transfected with the reporter construct were plated in 96-well plates (BioCoat™; Becton Dickinson, Franklin Lakes, NJ) at  $1 \times 10^4$  cells/well in 1% FBS alpha MEM (JRH Biosciences, Lexena, KS) containing L-Glutamine and sodium pyruvate (Life Technologies, Inc.). The cells were cultured for 24 hours, the medium was removed, and the wells were washed twice with wash buffer (1% BSA in PBS), 100 µl/well at 37°C. Zveg3, PDGF-AA, and PDGF-BB were diluted in assay medium (alpha MEM containing 1% BSA, 10 mM Hepes, L-Glutamate, and sodium pyruvate), and 100-µl samples were added to the wells. The plates were incubated 4 hours at 37°C, then the wells were washed with wash buffer. Lysis reagent (Cell Culture Lysis Reagent; Promega Corporation, Madison, WI) was diluted 1:5 in water, and 25 µl was added to each well. The plates were incubated 10 minutes at room temperature, then transferred to -20°C. Luciferase substrate (Luciferase Assay System; Promega Corporation) was diluted 1:1 with assay medium and added to the wells. The plates were read on a luminometer. Results, shown in Table 4, indicted that all three growth factors exhibited mitogenic activity on the CCC4 cells.

Table 4

Concentration (ng/ml)	Activity (Luminometer Units)		
	<u>Zveg3</u>	<u>PDGF-BB</u>	<u>PDGF-AA</u>
0	3.5	3.1	3.2
0.4	5.2	7.7	8.4
0.8	5.6	10.7	9.5
1.5	7.6	14	9.4
3.1	10.6	19.9	11.4
6.2	12.5	25.1	12
12.5	14.2	26.1	12.3
25	16	25.7	11.9
50	19.4	25.8	12.6
100	20.6	23.8	14

## 5 Example 12

A study was undertaken to test whether adenovirally delivered zveg3 stimulated cell proliferation as determined by incorporation of bromodeoxyuridine (BrdU) into tissues.

Mice (male, C57Bl, 7 weeks old) were divided into three groups. On day 0, parental or zveg3 adenovirus was administered to the first (n=11) and second (n=12) groups, respectively, via the tail vein, with each mouse receiving a dose of  $\sim 1 \times 10^{11}$  particles in  $\sim 0.1$  ml volume. The third group (n=8) received no treatment. Each mouse was given two intraperitoneal doses of 3 mg of freshly made BrdU solution at approximately 24 and 12 hours prior to sacrifice. On days 2, 4, 6, 8, and 10, two mice from each treatment group and one or two untreated mice were sacrificed, and tissues and blood were harvested. Samples were analyzed for complete blood count (CBC) and serum chemistry, and slides were prepared for manual differential blood and marrow progenitor cell analysis. One femur, lung, heart, thymus, liver, kidney, spleen, pancreas, duodenum, and mesenteric lymph nodes were submitted for standard histology and assessment of BrdU incorporation. The lining of the duodenum served as the control tissue for BrdU incorporation.

In addition, two mice that received approximately half the dose of zveg3 adenovirus particles and one mouse that received the full dose of parental adenovirus were sacrificed and analyzed as described above on day 16.

A piece of liver from each mouse was saved for mRNA assay of adenovirus protein to examine the time course of expression of the adenovirus preparations.

Beginning on day 6, most of the animals treated with either adenovirus had visibly enlarged livers and spleens compared to the untreated mice. The livers of



the zveg3 adenovirus-treated mice tended to look more pale than animals treated with the parental virus. Proliferation of sinusoidal cells was observed in liver. Visual inspection suggested that these cells were stellate cells and/or fibroblasts. Spleen color was the same in both groups. Most of the animals that received the zveg3 adenovirus had paler femur shafts, with the marrow lighter in color.

Peripheral blood CBCs showed a possible difference in platelet counts, but not in RBC or WBC counts between zveg3 and parental virus-treated animals. In comparison to the untreated and parental virus-treated groups, the zveg3 group had lower platelet counts on days 2, 4, 6, and 8, but not on day 10. The mean platelet volume (average size of individual platelets) in the zveg3 group also tended to be greater, consistent with a relative increase in the larger, immature platelet population.

BrdU labeling showed increased cell proliferation in kidney, mainly in the medulla and to a lesser extent in the cortex. Proliferating cells appeared to be interstitial cells, which may have included fibroblasts and/or mesangial cells.

#### Example 13

Human zveg3 growth factor domain protein produced in BHK cells was tested for the ability to stimulate production of TGF- $\beta$  in stellate cells. Rat hepatic stellate cells (obtained from Dr. Nelson Fausto, University of Washington) were plated in 48-well tissue culture clusters (Costar®; Corning, Corning, NY) in DMEM growth medium (Life Technologies, Inc.) supplemented with pyruvate and 10% serum (HyClone Laboratories, Inc.). At confluence, the medium was changed to serum-free medium by substituting 0.1% BSA (Fraction V; Sigma, St. Louis, MO) for serum. 48 hours later, the medium was changed, replaced with the same serum-free medium, and the zveg3 GFD protein was added at 100 ng/ml in the wells. 48 hours later, conditioned media were collected and spun down to get rid of any floating cells or debris. Total TGF- $\beta$ 1 levels were determined in these media using a commercially available ELISA kit (R&D Systems, St Paul, MN). Stimulation of stellate cells with 100 ng/ml zveg3 GFD resulted in an approximately 5-fold increase in the production of TGF- $\beta$  compared to a BSA control.

#### Example 14

Recombinant human zveg3 GFD was analyzed for mitogenic activity on human mesangial cells (Clonetics, San Diego, CA). Mesangial cells were plated at a density of 2,000 cells/well in 96-well culture plates and grown for approximately 72 hours in DMEM containing 10% fetal calf serum at 37°C. Cells were quiesced by incubating them for 20 hours in serum-free DMEM/Ham's F-12 medium containing

insulin (5 µg/ml), transferrin (20 µg/ml), and selenium (16 pg/ml) (ITS). At the time of the assay, the medium was removed, and test samples were added to the wells in triplicate. Test samples consisted of purified zveg3 GFD at 3, 10, 30 and 100 ng/ml, and PDGF-AA, PDGF-AB and PDGF-BB at 30 ng/ml in a buffer containing 0.1% BSA. Samples were serially diluted into ITS medium and added to the test plate. A control buffer of 0.1% BSA was diluted identically to the highest concentration of zveg3 protein and added to the plate. 10% fetal bovine serum (FBS) was used as a positive control. For measurement of [<sup>3</sup>H]thymidine incorporation, 20 µl of a 50 µCi/ml stock in DMEM was added directly to the cells, for a final activity of 1 µCi/well. After another 24-hour incubation, mitogenic activity was assessed by measuring the uptake of [<sup>3</sup>H]thymidine. Media were removed, and cells were incubated with 0.1 ml of trypsin until cells detached. Cells were harvested onto 96-well filter plates using a sample harvester (FilterMate™ harvester; Packard Instrument Co., Meriden, CT). The plates were then dried at 65°C for 15 minutes, sealed after adding 40 µl/well scintillation cocktail (Microscint™ O; Packard Instrument Co.), and counted on a microplate scintillation counter (Topcount®; Packard Instrument Co.). Results, shown in Table 5, demonstrated that zveg3 GFD had approximately 3-fold higher mitogenic activity cells over PDGF-AA and comparable activity to PDGF-AB and PDGF-BB at 30 ng/ml on mesangial cells.

Table 5

CPM [<sup>3</sup>H] Thymidine  
Incorporated

<u>Sample</u>	<u>Mean</u>	<u>St. dev.</u>
PDGF-AA, 30 ng/ml	492	120
PDGF-AB, 30 ng/ml	1554	114
PDGF-BB, 30 ng/ml	1852	464
Zveg3 GFD, 3 ng/ml	1321	91
Zveg3 GFD, 10 ng/ml	1615	325
Zveg3 GFD, 30 ng/ml	1545	237
Zveg3 GFD, 100 ng/ml	1677	88
10% FBS	1447	174
Buffer control	392	109

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration,

various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

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